Medicament for the treatment of diseases due to infection by *Neisseria Meningitidis*

The subject of the invention is a medicament for the treatment of diseases due to infection by *Neisseria meningitidis*, which comprises glycoconjugates and/or lipooligosaccharides (LOS) from commensal bacteria with cross-reactive antigens to *Neisseria meningitidis* and/or antibodies against such glycoconjugates and/or lipooligosaccharides.

Disease due to *Neisseria meningitidis* (NM) can kill a previously healthy child or young adult within hours of the first symptoms of illness. Meningococcal disease is the largest single cause of childhood death in the developed world. Worldwide over 350,000 fatalities caused by NM were registered by the World Health Organisation (WHO) per annum.

Meningococcal disease can manifest itself in two main forms, meningitis and septicaemia. Meningococcal meningitis is an inflammation of the meninges, the membrane lining the brain and the spinal cord. In both, fulminant meningococcal septicaemia and meningococcal meningitis damage is caused by an uncontrolled localised or systemic host inflammatory response.

Meningococcal septicaemia, or blood poisoning, is caused by invasion of meningococci into the blood system of the patient. The host's immune defence is unable to kill and clear the invading pathogen successfully, or to neutralize meningococcal toxins. During the evasion of the immune response or due to treatment with antibiotics, meningococci shed endotoxin or lipooligosaccharide (LOS) into the blood system. In the absence of specific or cross-reactive neutralising antibodies, endotoxin induces a massive

inflammatory response characterised by increased secretion of inflammatory mediators such as interleukin 1 (IL-1), interleukin-6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF α), interferon gamma (IFN γ) and acute phase proteins. Severity and fatality of the disease has been correlated with levels of inflammatory mediators detected in the blood.

In susceptible patients, this release of endotoxin and the results of inflammatory responses lead to rapid deterioration and failure of the normal homeostatic mechanisms. The removal of free endotoxin and the intervention in controlling the inflammatory response to endotoxin are crucial in preventing further damage to the host. Disseminated intravascular coagulation or blood platelet aggregation can result in the loss of limbs. Bleeding or leaking of peripheral blood into the surrounding tissue of blood vessels is recognised by the typical spots under the skin. The loss of perfusion may lead to the patient falling into a coma. Myocardial depression and multiple organ failure can lead to death.

Because meningococci are transmitted by aerosols or close ("kissing") contact, immunisation is the only effective means for prevention of disease in individuals lacking protective immunity.

NM is an exclusively human pathogen. It is Gram-negative, 1µm in diameter, aerobic diplococcus and shows a large degree of phenotypic variation.

The major antigens of the outer membrane of meningococci vary greatly and these variations have been exploited to develop typing systems for epidemiological surveillance. These include capsular polysaccharide, a variety of outer membrane proteins, pili and endotoxin. The main antigens are found anchored to the typical Gram-negative envelope (Figure 8).

The first major success in development of vaccines against meningococcal disease was through the recognition of the immunogenicity of the capsular polysaccharide of these bacteria.

Originally meningococci were divided into serogroups for epidemiological purposes based on agglutination of capsular antigens. Currently 12 major antigenically distinguishable polysaccharide capsules have been identified: A,

B, C, H, I, K, L, X, Y, Z, 29E and W135 which vary in their composition and arrangements of oligosaccharide units. The most prevalent serogroup structures are presented in table 1 The majority of meningococcal disease is caused by serogroup A, B and C. Groups B and C are responsible for most disease in Europe and the Americas while group A is more prevalent in Africa, Russia and causes periodic epidemics in Romania.

Table 1: Oligosaccharide structures of the major pathogenic meningococcal polysaccharide capsules

Sero-group	Capsular Polymer	
A	*N*-acetyl-3-0-acetyl mannosamine phosphate (α1→6), (*O*-acetylated-2-acetamido-2-deoxy-D-mannose-6-phosphate)	
B	Up to 200-residue polysaccharide units of (2→8) linked *N*-acetylneuramic acid	
C	O-acetylated or non acetylated (2→9) linked N-acetylneuramic acid	
X	*N*-acetyl glucosamine phosphate (α1→4), or (2-acetamido-2dedoxy-D-glucose-4-phosphate)	
Y	*N*-acetyl neuraminic acid:glucose, partially *O*-acetylated alternating sequences of D-glucose and *N*-acetylneuramic acid	
W-135	4-*O*-α-D-galactopyranosyl-β-D-N-acetyl-neuraminic acid, alternating sequences of D-galactose and N-acetylneuramic acid	

Polysaccharide capsules are an effective way for pathogens to evade the human immune responses. Compared with non-capsulate meningococci, which are usually eliminated by bactericidal and opsonising antibodies in human serum, heavy polysaccharide capsulation is thought to reduce the ability of complement to bind and kill meningococci. Group B meningococci express a poorly immunogenic α2→8 linked poly-sialic capsule similar to some human

antigens such as the Neural Cell Adhesion Molecule (N-CAM).

Capsular antigen vaccines

Capsular polysaccharide vaccines against the serogroups A, C, Y, and W-135 induce protective immunity against these meningococcal serogroups in older children and adults. While these vaccines appear to be effective in adults, vaccines based on meningococcal polysaccharides are less effective in young children. Group C vaccines are thought to be ineffective in children younger than 2 years of age, and in children under 6 months for group A vaccines. The duration of protection elicited by capsular antigens is thought to be short lived, varying between two to four years after administration of the vaccine in adults and children.

This lack of wide-scale protection within the young age group (6 months to 5 years) that is most susceptible to meningococcal disease led to the development of conjugated group C vaccine. The principal is that used for the successful development of a vaccine for *H. influenzae* type b (Hib) in which the polysaccharide was conjugated to a carrier protein. Conjugation of polysaccharides to protein carriers induces a T-cell dependent response compared to polysaccharide alone which induces a T-cell independent response. Large molecular weight polysaccharide antigens like the meningococcal capsule bind to several receptors on B cells followed by cross-linking of these receptors. This triggers the production of immunoglobulin IgM and the transformation of the stimulated B cell into plasma cells. This T-cell independent immunity is short lived and does not generate memory.

The protein-carbohydrate antigen is ingested by antigen presenting cells (APC) and expressed on their cell surface within the major histocompatible complex type II receptor (MHC II). T-helper (T_H) cells expressing the MHC II receptor (CD4) and CD28 (B7 receptor) are activated by the APC leading to clonal proliferation and T_H cell maturation with some developing into memory T-cells. The activated T_H cells lead to differentiation of B-cells that bind directly to the hapten, *i.e.* a second encounter with the antigen or antigen present on the APC, followed by proliferation of B-cells, their differentiation into antibody producing plasma cells, or memory B-cells. This T-cell dependent immunity is

long lasting and able to produce a wide range of classes of immunoglobulin. A serogroup C conjugate vaccine was introduced as part of a mass vaccination program in the United Kingdom in the autumn of 1999. Initial observations on the effectiveness of the conjugated vaccine in Scotland following mass vaccination of children and young adults indicate a reduction in disease caused by group C meningococci.

The remaining problem of serogroup B

While the conjugate C vaccine appears to have partly reduced disease due to this serogroup, the NeuNAc capsule of group B meningococci is thought to be ineffective due its low immunogenicity and its presence on some human tissues (*i.e.*, neural cell adhesion molecule, N-CAM). Poly-sialylated N-CAM is an antigen found in several tumours associated with the immune evasion of some malignant metastatic cells. Protein vaccines containing the B capsular antigen did not show such effects in animal models or in humans. Vaccines based on group B capsular polysaccharide are poorly immunogenic, and short lived. It rarely induces antibodies in patients. Attempts to increase the immunogenicity by conjugation with protein carriers were not successful.

Because of the problems outlined above, other surface antigens of meningococci have been assessed for their use as vaccine candidates for serogroup B meningococci.

While pili are associated with colonisation but not with invasive disease, the pilus antigens have not been assessed as vaccines because of the hypervariability of their terminal protein sequences.

Neisseriae species have a typical Gram-negative envelope consisting of a lipid bi-layer around a semi-rigid peptidoglycan sheet. Proteins can be anchored to the outer lipid layer alone, but usually form monomeric or polymeric structures penetrating both lipids and peptidoglycan layers (trans-membrane OMP).

Five classes of outer membrane proteins have been identified in NM. The classification is based on the molecular weight of proteins separated by sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis. Monoclonal antibodies to class 2 and class 3 outer membrane proteins have been used in

epidemiological typing of meningococci (serotype), as have monoclonal antibodies to class 1 to determine the subtype. Class 1 OMP are porins selective for cations and are expressed on meningococcal isolates obtained from carriers and patients. There is antigenic variability within the class 1 OMP which has been used to develop monoclonal antibodies used in epidemiological surveillance. Investigations into the ability of OMP to elict antibodies showed that deglycosylation of all investigated classes (1-5) resulted in no significant antibody production *in vivo*. This suggests that antigenicity of OMP depends on post-translational glycosylation, or the presence of other oligosaccharides that must be considered in evaluation of these surface components for their use in vaccines.

In addition to the five major classes of outer membrane proteins, several other molecules are present on the meningococcal surface. Several iron binding OMP with variable molecular weights are currently under investigation as potential vaccine candidates for meningococci

Outer membrane vesicle (OMV) vaccines

Various vaccines derived from deglycosylated OMV were tested in animal models and in clinical trials in Norway, Brazil, Cuba and Chile. These vaccines induced bactericidal antibodies in the immunised group but were protective mainly against strains expressing the serotype/subtype antigens of the strain from which the vaccine was produced. There was limited protection against strains expressing other OMP antigens. Due to the heterogeneity of antigens on meningococcal strains and the introduction of new strains into the vaccinated population, the use of OMV would only be effective in closed populations (*e.g.*, Cuba).

Lipooligosaccharides (LOS)

All Gram-negative species have a family of glycolipids called endotoxin embedded into the hydrophobic outer membrane lipid layer. These macromolecules share a common basic structure consisting of :

- a basal lipid A region anchored into the outer membrane;
- a rough (R) core region consisting of a backbone of 2-keto-3-

deoxyoctulosonic acid (KDO) and/or heptose (Hep) phosphate;

- a highly variable region of saccharide domains differing in length and composition bound to the core heptose residue.

Lipid A is a phosphorylated di-glucosamine disaccharide substituted with fatty acids of variable length, and it is responsible for the biological activity which induces inflammation (endotoxin). Enteric Gram-negative species show a characteristic long linear chain of polysaccharide, called O-antigen, linked to the R-core giving the endotoxin of these species the name lipopolysaccharide (LPS). In contrast, the saccharide chains of all *Neisseria* species consist of very few residues, giving its endotoxin the name lipooligosaccharide (LOS).

De-glycolysated OMV vaccines showed poor immunogenicity after the removal of its toxic LOS moieties. LOS appears to be an essential component of anti-meningococcal protein vaccines, perhaps acting as an adjuvant in the human host.

Variation in meningococcal LOS and immunotyping

Thirteen major LOS types were identified for *N. meningitidis* using polyclonal and monoclonal antibodies by passive haemagglutination inhibition techniques and whole cell ELISA. The majority of meningococcal isolates express one or more of the immunotypes L1-L12, while non-typable and L13 immunotypes are rare. The twelve major LOS types have a relative molecular weight ranging from 3.15 to 7.1 kDa. The oligosaccharide chain, also referred to as the α-chain or variable LOS region one (R1), is composed of the saccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc). Sialylated forms contain the terminal saccharide N-acetylneuramic acid (NeuNAc) which is added to terminal galactose residues by endogenous or exogenous sialyl transferases. Abbreviations for core moieties are used as follows: glycero-D-manno-heptopyranoside (Hep or heptose); phospho-ethanolamine (PEA); 2-keto-3-deoxyoctulosonic (KDO).

The complete structures of immunotypes L10 - L13 are not elucidated, but there is evidence that L10 contains the paragloboside residue and L11 shows some homology with L1. The PEA residue of immunotype L2 can be expressed

in two forms that undergo phase variation: The PEA on the G3 region can be linked in (1→6) or (1→7) conformation; and the PEA can be replaced by a hydrogen (H) atom. The PEA residue of immunotypes L4 and L6 express both PEA (1→6) and (1→7) linkages. The expression of meningococcal immunotypes is associated with serogroups. Immunotypes L8, L9, L10, L11 and L12 are found on group A strains, while serogroup B and C meningococci express immunotypes L1 - L8.

Immunotypes and pathogenicity

LOS immunotype expression is thought to be linked to the pathogenicity of the organism. Immunotypes L(3,7,9) are isolated predominantly from patients with invasive meningococcal disease. Other immunotypes are found predominantly among carrier strains. Immunotypes L3, L7 and L9 are thought to be similar in their immunochemical structures with immunotype L3 being sialylated by endogenous sialyl transferases. Immunotypes L3 and L7 are found on serogroup B and C meningococci and they have similar G2 core components, PEA (1→3) HepII. Immunotype L9 is expressed on group A strains.

The presence of the sialylated phenotype on invasive meningococci is associated with resistance to complement-mediated killing by masking the terminal galactose with NeuNAc. This mechanism is thought to reduce the recognition of the epitope by anti-LOS antibodies directed against the non-sialylated epitopes. Free or membrane bound sialyl-L(3,7,9) also upregulates neutrophil activation markers and results in increased injury of epithelial cell lines. Sialyl L(3,7,9) phenotypes can evade the complement mediated bacteriolysis cascade. This phenotype also reduces complement and anti-LOS antibody mediated phagocytosis by professional phagocytes.

Expression of major and minor immunotypes by *N. meningitidis*

Meningococci are able to express more than one immunotype. Isolates from patients with meningococcal disease in the Netherlands (1989-1990) showed different immunotype combinations (Scholten R.J., Kuipers B., Valkenburg

H.A., Dankert J., Zollinger W.D., and Poolman J.T. (1994), *J.Med.Microbiol.* **41**(4):236-43).

1. Group A meningococci L**9** (54%), L**9,8** (8%), L**10** (24%), L**10,11** (8%) and non-typable (**NT**) (8%).
2. Group B meningococci L**1** (1%), L**1,8** (11%), L**2** (10%), L**3,7** (36%), L**3,7,1** (4%), L**3,7,1,8** (2%), L**3,7,8** (28%), L**4** (4%), and L**8** (5%).
3. Group C meningococci L**1,8** (2%), L**2** (30%), L**3,7** (37%), L**3,7,1** (1%), L**3,7,1,8** (3%), L**3,7,8** (7%), L**4** (15%), L**8** (3%), and **NT** (3%).

The expression of multiple immunotypes within a meningococcal population allows the organism to diversify its antigenic structure. Selective pressure due to the presence of antibodies in the host to one LOS immunotype allows the strain to express other immunotypes increasing their chance of survival. This ability of meningococci to alter its LOS structure has to be taken into account in understanding the development of natural immunity, and in the choice of immunotypes as potential vaccine candidates. Sialylation and the expression of paragloboside gene cluster IgtABE are the main phase variable phenotypes known.

The expression of meningococcal immunotypes undergoes phase variation due to *in vitro* growth conditions. The variability of meningococcal phenotypes and LOS expression depends on the growth rate and phase, as well as the presence of exogenous sialyl transferases.

Structural homology between LOS and human blood group antigens

Some LOS residues mimic human blood group antigens (Table 2).

Table 2: Homology of human blood group antigens with meningococcal LOS residues.

oligo-saccharide:	α chain moiety:
P1 blood group	Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ

p^k, CD77	Galα (1→4) Galβ (1→4) Glcβ
P globoside	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ
Para-globoside	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i a determinant	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ(1→3) Galβ (1→4) Glcβ
i b determinant	Sialyl-Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ(1→3) Galβ (1→4) Glcβ
Cer-dihexocide	Galβ (1→4) Glcβ

Gal, galactose; GlcNAc, N-acetylglucosamine; Glc, glucose; Sialyl, sialyc acid; cer, ceramide; the Glcβ on the reduced end is linked to (1→1) ceramide

The G1 regions of L1 and L11 LOS show identical terminal oligosaccharide residues of ceramide trihexocide, the p^k blood group antigen (CD77, globoside). The lacto-N-neotetranose of immunotypes L2, L(3,7,9) and L5 are identical to paragloboside, a precursor of P1 blood group antigen found in 75% of Caucasians, and the 3 terminal sugars are present in the Ii antigen. Immunotype L6 shares its two terminal sugars with the P blood group antigen, and L8 shares its terminal disaccharide with the common precursor of the P blood group system and steroid receptors. Both blood group antigens and meningococcal LOS with a terminal galactose residue can exist as sialylated and non-sialylated forms

LOS vaccines

Meningococcal LOS vaccine induce a strong and potentially fatal inflammatory response due to the toxicity of its lipid A component. The oligosaccharide from which lipid A has been removed is not immunogenic. Meningococcal LOS is closely associated with the severity and fatality of disease. This is mainly due to its involvement in inducing large amounts of pro-inflammatory cytokines in a CD14 dependent mechanism. Anti-meningococcal LOS antibodies are not only bactericidal, but also opsonising in nature, resulting in the phagocytosis of invading bacteria and LOS containing blebs by human monocytes. Normal

human serum of adults usually contains antibodies against meningococcal LOS, suggesting its important role in development of natural immunity to meningococcal disease.

The problem underlying the present invention is to provide new highly effective therapeutics and vaccines for diseases caused by *N. meningitidis*. A problem is especially to provide effective theurapeutics and vacchines agaist serogroup B, which are not available. The vacchines should be highly immunogenic, have a long-lasting effect and have low levels of toxicity, being therefore safe and effective in children and adults.

Surprisingly, the problem is solved by a medicament for the treatment or prevention of diseases due to infection by *Neisseria meningitidis*, characterized in that it comprises glycoconjugates and/or lipooligosaccharides (LOS) included in outer membrane vesicles, blebs, lipid layers, liposomes and/or killed or commensal bacteria with cross-reactive antigens to *Neisseria meningitidis* of the serogroup A, B, C, H, I, K, L, X, Y, Z, 29E or W135, or non-capsulated meningococcal strains and/or antibodies against such glycoconjugates and/or lipooligosaccharides.

Explanation of the Figures:

Figure 1: Schematic structure of meningococcal LOS immunotypes G3: PEA (1→6) L2, L4, L6; G2: PEA (1→3) L1, L8, L(3,7,9); H (→3) L4, L6; αGal (1→3) L2

Figure 2: TNFα (IU ml^{-1}) responses to LOS from meningococci (L3, L6), *N. lactamica* 1 or *E.coli* endotoxin (100 pg ml^{-1}) by (a) undifferentiated, (b) differentiated THP-1 cells (n=6, error bars = SD)

Figure 3: IL-6 (pg ml^{-1}) responses to LOS of meningococci (L3, L6), commensal isolates *N. lactamica* 1 or *E. coli* endotoxin (100 pg ml^{-1}) by (a) undifferentiated, (b) differentiated THP-1 cells (n=6, error bars = SD)

Figure 4: (a) Release of TNFα (IU ml^{-1}) from VD3 differentiated THP-1 cells challenged with meningococcal LOS, *N. lactamica* 1 LOS or *E. coli* LPS (100 pg ml^{-1}), (b) endotoxins co-incubated with pooled human serum (final dilution 1 in 1000), (c) endotoxins co-incubated with immune mouse serum produced by

vaccination with *N. lactamica* 1 (final dilution 1 in 1000) (n=6; error bars = standard deviation)

Figure 5: (a) Release of IL-6 (pg ml^{-1}) from VD3 differentiated THP-1 cells challenged with meningococcal LOS, *N. lactamica* 1 LOS or *E. coli* LPS (100 pg ml^{-1}), (b) endotoxins co-incubated with pooled human serum (final dilution 1 in 1000), (c) endotoxins co-incubated with immune mouse serum produced by vaccination with *N. lactamica* 1 (final dilution 1 in 1000) (n=6; error bars = standard deviation)

Figure 6: TNFα (IU ml^{-1}) responses to LOS from meningococci (L3, L6), commensal species (NL1, MC1, MC2) or *E.coli* endotoxin (100 pg ml^{-1}) by differentiated THP-1 cells (n=6, error bars = SD)

Figure 7: IL-6 (ng ml^{-1}) responses to LOS of meningococci (L3, L6), commensal isolates (NL1, MC1, MC2) or *E. coli* endotoxin (100 pg ml^{-1}) by differentiated THP-1 cells (n=6, error bars = SD)

Figure 8: Capsular polysaccharide, outer membrane proteins (OMP) and transmembrane proteins, pili, lipooligosaccharide (LOS) of meningococci.

Figure 9: (Opsono-) Phagocytic and anti-inflammatory mechanisms during meningococcal disease.

Figure 10: Effect of trypan blue quenching on the mean ingestion index after 15 min incubation with opsonised PI-labelled *M. catarrhalis* strains or neisseriae strains.

Figure 11: The effect of antibody and complement on ingestion of (a) NL1 and (b) L7 meningococci by THP-1 cells (mean of six independent experiments)

Figure 12: Percentage inflammatory response of human monocytic cell line THP-1 challenged with meningococcal endotoxin immunotype L(3,7,9) (1 ng 10^5 cells) in the presence of cross-reactive antibodies obtained from different sources in the absence and presence of sodium-selenite (10 µg mL^{-1}).

WO-A-00/50074, EP 0 941 738 and Ji Yin-Duo "ZHONGHUA WEISHENGQUXUE HE MIANYIXUE ZAZHI, vol. 14, no. 4, 1994 pages 223-237 describe the induction of potentially bactericidal antibodies to endotoxins from

meningococci and N. lactamica. There is no other evidence presented that other functional antibodies, particularly cross-reactive antibodies to lipooligosaccharides with anti-inflammatory activity, as disclosed in the present invention is present. The anti-inflammatory and opsono-phagocytic activity of cross-reactive antibodies against our vaccine and treatment candidates is novel (i.e. Table 18).

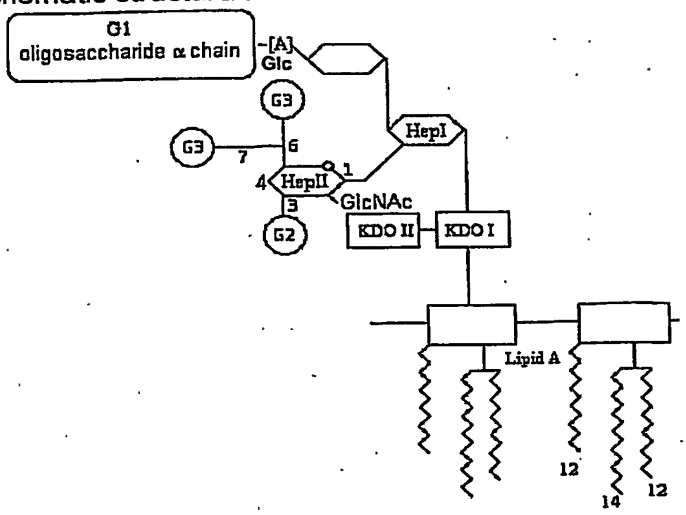
Griffiss *et all* (Transactions of the Royal Society of Tropical Medicine and Hygiene, 1991) discloses the use of inner core antigens of meningococcal LOS as a target for potential vaccine development, and postulates that the blood group like antigens are potential self antigens and therefore not immunogenic in humans, or that these antigens do not induce functional antibodies, and thus, are not involved in the development of immunity against meningococci. The present invention challenges this view. Anti-blood group like substances might not induce bactericidal antibodies, but homologous and heterologous anti-blood group antibodies (i.e. Ii, paragloboside, P, pK) show a strong anti-inflammatory and opsono-phagocytic potency in vitro (i.e. Figure 12) against meningococci and commensal bacteria sharing these blood group like antigens. In particular, the invention provides a novel approach in so far, that functional antibodies are induced.

All the data presented by Ji Yin-Duo are based on bactericidal antibodies against cross reactive LOS antigens most likely directed against inner core LOS epitopes, and not the outer core glyco- structures (see Griffiss above).

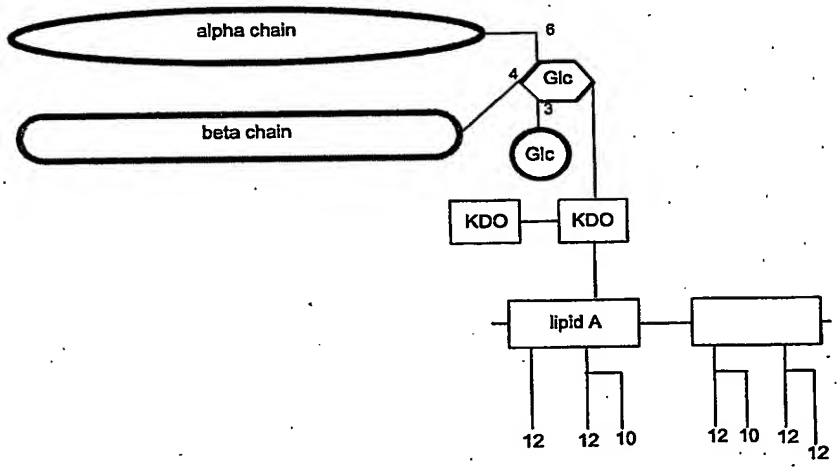
EP-A-0 941 738 discloses homology between neisseria species and M. catarrhalis by providing evidence that antigens obtained from LPS from these species were covalently bound to a protein carrier, including membrane proteins from N. meningitidis and M. catarrhalis [0037]. The present invention does not include cross-reactivity of proteins between these species. Further it is disclosed, that antibodies can be used in the tratment or prophylaxis of septic shock caused by bacterial endotoxins [0043-0044] using antibodies to the comman core stucture endotoxin from these bacteria.

But M. catarrhalis and N. meningitidis do not share a common core structure or the same lipid A moieties (Figure 4) [Holme *et al.*, (1990) The lipopolysaccharide of Moraxella catarrhalis structural relationships and antigenic properties. *Eur. J. Biochem.* 265(2): p. 524-529]. N. meningitidis Lipid A comprises of one single strand fatty acid with 12 carbon molecules (C), and a second with 14 C branched with a 12 C strand. M. catarrhalis contains a structurally different Lipid A with one molecule showing a single C12, and a branched c12:c10 strand, while the second Lipid A molecules comprises of one C12:C10 and one C12:C12 branched strand. The only structural homology of LOS core structures between the two species are the two KDO molecules attached to Lipid A. The inner core of LPS do not share homology. While *N. meningitidis* LPS show a heptose molecule attached to the first KDO as the base of the inner core structure, this structure is absent in *M. catarrhalis* LOS where a Glucose molecule is present. Furthermore, the second Heptose molecule found in N. meningitidis is also absent in *M. catarrhalis* LOS. This allows to postulate that the described homology between the described species is based on the GlcNAc- KDO structure bridging the Lipid A moieties and the inner core LOS structure as disclosed in paragraph 0044. The reference provides evidence in the molecular structures of Figure 1, 2, and 3. This homology of the GlcNAc-KDO epitope is further supported by the experimental evidence shown in Figure 4 of description in the reference. Accepting this evidence, the reference does not challenge the teaching of structural homology of these bacteria species based upon the outer core (blood group like) antigens inducing functional cross-reactivity.

Schematic structure*N.* LOS



Schematic structure*M.* LOS



WO-A-00/50074 A3 relates to vaccines that provide a broad spectrum protective immunity to microbial infection. It discloses that their vaccine protects against all or a wide range of strains (p. 2 paragraphs 25- 32). The commensal bacterium species N. lactamica was used as a live vaccine or a killed whole cell vaccine or a vaccines containing fractions of N. lactamica. (p.3 paragraphs 8-24) or other neisseria species (p. 4 paragraphs 1-5). The reference discloses that the vaccine contains outer membrane molecules including lipooligosaccharides (p. 5 §19- p. 6 §18). The described vaccine is produced by extracting outer membrane vesicles using a detergent. It is well known that this method removes the lipooligosaccharide molecules from the

outer membrane. This detergent treated vaccine is considered to be endotoxin free and therefore has no effect on the teaching of the present invention.

Using LOS based vaccines (Example 3, p. 17 paragraphs 25-32) of WO-A-00/50074 and consequent immunisation of mice using this LOS vaccine (Example 4, p. 18 paragraphs 1-22) showed that this LOS vaccine did not protect immunized mice against meningococcal LPS challange. As the reference discloses, *„... all members of the control group and of the group vaccinated with LOS (marked LPS on the figure) had died.*" (p. 18 §21-22). Only protein vaccines (with detergant removed endotoxin) was effective in protecting mice against meningococci. The alleged teaching to have developed a vaccine with cross-reactive LOS antigens between commensal neisseria species and N. meningitidis, can not be upheld when the description present evidence that LOS vaccine according to the reference was not protective.

In a preferred embodiment of the invention, the medicament is a vaccine. The use of LOS from commensal bacteria as a vaccine has several advantages over other vaccines. The most common meningococcal LOS immunotype associated with disease is L(3,7,9) found in both group B and C outbreak strains of meningococci in Europe and America. The anti-meningococcal vaccine obtained from commensal bacteria which comprise glycoconjugates and/or LOS cross-reactive with meningococcal LOS including immunotype L(3,7,9) is effective against more than 90% of meningococcal outbreak strains worldwide. The LOS is highly immunogenic in all age groups, including young children, leading to long lasting protective immunity.

"Commensal" means species of *Neisseria* or closely related species, which are not *Neisseria meningitidis*, with a human host. The commensal bacteria with cross-reactive antigens to *Neisseria meningitidis* are preferably *Moraxella catarrhalis* (MC) or *Neisseria lactamica* (NL).

N. lactamica is a non-pathogenic commensal, rarely reported to cause disease in humans. The structures and expression of LOS of NL appear to be as diverse as those of meningococci. Kim *et al.* (Kim J.J., Mandrell R.E., and

Griffiss J.M. (1989) *Infect.Immun.* **57**(2): p. 602-608) identified epitopes common to NL which were recognised by two monoclonal antibodies produced against NM. The antibody D6A bound to meningococcal immunotypes L1, L8, L(3,7,9), L10 and L11. The antibody 06B4 bound to L2, L4, L8 and L(3,7,9) immunotypes.

None of these immunotypes share any of the P-blood group related saccharides of the G1 terminal α-chain of meningococcal LOS. Later analysis of the LOS structure revealed that these immunotypes share a common core structure, the G2 and G3 region of the second core heptose (HepII). The third carbon shares a hydroxyl (-OH) group, and the fourth carbon contains a PEA residue.

M. catarrhalis (MC) is a commensal Gram-negative diplococcus previously classified within the genera *Branhamella* and *Neisseria.* Recent genetic studies resulted in the re-classification of MC into the genus *Moraxella*.

Although MC is associated with some childhood diseases, it is frequently isolated as a commensal from the respiratory tract in healthy young children. Children are colonised with 3-4 different strains of MC within the first two years of life. It is isolated more frequently than *Neisseria* species during the first 6 months of life when infants are developing antibodies to the bacteria in their environment. One member of the genus, *Moraxella nonliquefaciens* expresses a capsular antigen similar to group B meningococci and *E. coli* K1, and it is isolated from about 20% of healthy carriers.

Several surface antigens are thought to be involved in the development of immunity to MC. Carriage and infections with MC are associated with the development of protective IgG from an early age. These include protein antigens and glycoconjugates. Two OMP are associated with protective immunity, UspA1 and UspA2, possibly due to structural homology and cross-reactivity detected with monoclonal antibodies.

Although LOS from MC differs structurally from meningococcal LOS, both species share some homology in their oligosaccharide chain moieties. Terminal oligosaccharide residues found on the non-reducing end of MC LOS share some homology with human blood group antigens. Combination of five

different saccharide residues of the α or β chains determine the immunotype of MC LOS (Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; KDO, 2-keto-3-deoxyoctulosonic):

1. Galα (1→4) Galβ (1→4) GlcNAcα (1→2) Glcβ;
2. Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ;
3. GlcNAcα (1→2) Glcβ;
4. Glcα (1→2) Glcβ;
5. Glcβ.

Preferably, the medicament of the invention is used for the treatment or vaccination for diseases caused by *Neisseria meningitidis* of the serogroup A, B, C or W135. The use against serotype B is of high significance, because the LOS of the commensal species, especially NL and MC, are of low toxicity in contrary to those of NM. Therefore, the medicament and vacchine of the invention allow for the first time an effective treatment and vacchination of diseases caused by serogroup B NM.

The glycoconjugates or lipooligosaccharides may be included in outer membrane vesicles, blebs, lipid layers, liposomes or killed or viable bacteria commensal to *Neisseria meningitidis.* Preferred Glycoconjugates used according to the invention are lipooligosaccharides and glycolipids. It is also advantageous to apply glycoconjugates, which are glycoproteins. Preferrably, immunogenic sugar moieties are conjugated to protein carriers.

In a preferred embodiment of the invention, the glycoconjugates or lipooligosaacharides are chemically modified, conjugated or hydrolized, preferably by mild acid hydrolysis. Preferably, mild acid hydrolysis is applied under conditions such that fragments of lipooligosaccharides are obtained.

The lipid A moieties, core, and oligosaccharide antigens that can be obtained through chemical modification of LOS from NL and/or MC induce cross-reactive, bactericidal, opsonophagocytic and anti-inflammatory (functional) antibodies. Further, genetic modification of the genes encoding the lipid A, core and oligosaccharide expression and assembly can be used to produce a native and/or structurally defined LOS. The use of glycosyltransferases associated with LOS synthesis and assembly onto a protein carrier, or (detoxified) lipid A, or liposome carrier allows the production of a synthetic molecule able to mimic and/or induce cross-reactive functional antibodies to be used as a vaccine and/or a medicament for the treatment of meningococcal disease.

Hydrolysis may for instance be performed in a way that immune-accessible glyco- or lipid A epitopes are obtained.

The antibodies which are part of the medicament of the invention may be monoclonal or polyclonal and of animal or human origin. Advantageously, they are obtained

from virus immortalized human lymphocytes secreting the glycoconjugate neutralizing, specific or cross-reactive antibodies,

from human lymphocytes secreting the neutralizing antibodies fused with a human hybridoma cell line, or

from immunized animals, preferably mice, rats, rabbits or pigs, producing polyclonal serum containing such antibodies, or

from immunized animals, preferably mice, rats, rabbits or pigs, after fusion of the animal lymphocytes with a human or animal hybridoma cell line.

If the medicament of the invention comprises such antibodies, it is preferably used for the treatment of acute meningitis or septicaemia as an anti-inflammatory, bactericidal and/or opsonophagocytosis inducing medicament.

The medicament of the invention is advantageously applied as a nasal or oral spray, as a liquid for injection, as an orally applied capsule or as a tablet. It may be applied in combination with an adjuvant.

A subject of the invention is also a diagnostic to assess the susceptibility of patients for diseases due to *Neisseria meningitidis*, which comprises glycoconjugates and/or lipooligosaccharides from from commensal bacteria with cross-reactive antigens to *Neisseria meningitidis* or antibodies against such glycoconjugates or lipooligosaccharides.

Examples

Bacterial strains

Standard immunotype strains of meningococci L1-L12 were obtained from Dr. W. D. Zollinger, Washington D.C. NL isolates were obtained from our culture collection. None of the isolates were agglutinated by standard serogroup reagents and none reacted with the monoclonal antibodies used to determine serotype or subtype of meningococci.

Cultures were grown overnight at 37°C on human blood agar (HBA) containing: lysed whole blood (100 ml) from the Scottish National Blood Transfusion Service (SNBTS); special peptone (23 g) (Difco); corn starch (1g) (Sigma); NaCl (4.5 g) (Sigma); D-glucose (1 g) (Sigma); technical grade agar (10 g) (OXOID); K_2HPO_4 (4 g) and KH_2PO_4 (1 g); 900 ml of distilled water.

Bactericidal Assay

The microtiter plate method described by Zorgani *et al.* (Zorgani A.A., James V.S., Stewart J., Blackwell C.C., Elton R.A., and Weir D.M. (1996), *FEMS Immunol Med Microbiol.* **14**(2-3): p.73-81) was used to screen for bactericidal activity.

A pool was prepared with serum from eight healthy adult donors with no known history of meningococcal disease. The pool was inactivated at 56°C for 30 min, divided into aliquots which were absorbed twice at 4°C overnight with viable individual strains of NL (10^{10} bacteria ml^{-1}), centrifuged at 1000 x *g* and filter sterilized using a 0.22 µm membrane (Nu-flow, Oxoid). Aliquots of the absorbed sera were tested for sterility and stored at -70°C.

The complement source was prepared from a blood sample from a healthy adult volunteer with no known history of meningococcal disease. Serum was supplemented with 1mM EDTA to ensure the bactericidal activity observed reflected the classical antibody-mediated complement killing and not the alternative pathway. The serum was absorbed twice with a pool of the meningococcal test strains grown overnight at 37°C in a humidified atmosphere with 5% (v/v) CO_2 on HBA. The absorbed serum was sterilized through a 0.22 µm membrane filter. The complement source was tested for sterility and stored in aliquots at -70°C. Complement titres were assessed with sensitised sheep red blood cells and used in the assays at a dilution of 1 in 16.

For testing in the bactericidal assay, strains were grown overnight on HBA and washed twice in PBS by centrifugation at 1000 x *g*. The total count for each strain was determined microscopically with a Thoma counting chamber and adjusted to approximately 10^5 colony forming units (cfu) per ml in sterile PBS containing $MgCl_2$ (0.5 mM), $CaCl_2$ (0.9 mM) and glucose (0.1%, w/v) (Sigma) (pH 7.2).

Triplicate samples containing equal volumes (40µl) of the test strain (approximately 400 cfu/well) and the heat inactivated serum pool were incubated with 20 µl of the complement source for 30 min in sterile U-bottomed 96 well microtitre plates. Three drops (10 µl) from each sample well were placed on HBA plates which had been dried for 48 hours at room temperature. The plates were incubated overnight at 37°C, the mean cfu recorded and used to calculate the serum bactericidal activity. Each assay included two controls: 1) bacteria + complement source + D-PBS but no serum; 2) bacteria + heat inactivated complement source + absorbed or unabsorbed heat inactivated serum pool.

The absorbed and unabsorbed pools were tested in parallel and the bactericidal activity of the absorbed and unabsorbed pools were compared. Compared with results obtained with the unabsorbed serum, reduction in bactericidal killing ≥ 80% with the absorbed serum was taken as evidence that the NL strain had removed significant levels of bactericidal activity.

Assay for inflammatory responses

The human monocytic cell line THP-1 was obtained from the European Collection of Animal Cell Cultures (ECACC, UK). Cells were grown to 10^4 to 10^6 cells ml^{-1} in RPMI-1640 cell culture medium (Sigma, Poole, Dorset, UK) supplemented with foetal calf serum (FCS) (5%, v/v) (Gibco), L-glutamine (1%, w/v) (Gibco), penicillin (100 IU ml^{-1}) and streptomycin (200 mg ml^{-1}) (Gibco) for not more than 18 weeks after establishing the cell line. The calf serum did not contain antibodies against any of the bacterial strains tested as determined by whole cell ELISA (Scholten R.J., Kuipers B., Valkenburg H.A., Dankert J., Zollinger W.D., and Poolman J.T. (1994), *J.Med.Microbiol.* **41**(4):236-43).

The mouse fibroblast cell line L929 was obtained from the ECACC. Cells were grown in 75 cm^3 tissue culture flasks (Greiner) to 70 % confluence in growth medium containing DMEM medium (Sigma) supplemented with FCS (5%, v/v) (Gibco), L-glutamine (1%, w/v) (Gibco), penicillin (100 IU ml^{-1}) and streptomycin (200 mg ml^{-1}) (Gibco) at 37 °C with 5% CO_2 (Gordon A.E., Al Madani O., Weir D.M., Busuttil A., and Blackwell C.C. (1999), *FEMS Immunol Med Microbiol.* **25**(1-2): p. 199-206).

Extraction of LOS

All strains were grown for 18 h in 5% (v/v) CO_2 on HBA. Cells were harvested from plates, washed in sterile pyrogen free PBS, centrifuged at 1000 x *g* and resuspended in pyrogen free distilled water. The hot phenol-water method described by Hancock and Poxton (1988, Modern Microbiological Methods: Bacterial Cell Surface techniques John Wiley & Sons, Chichester, UK) was used to extract the LOS. The purified LOS contained protein contaminants of <1% (w/w) as assessed against a standard of bovine serum albumin (BSA) (Sigma). LOS was resuspended in RPMI-1640 medium (Sigma) and sterilized through a 0.22 µm membrane filter. Aliquots were stored at -70° C and two samples from each batch were incubated at 37° C for 18 h to test for sterility.

Induction of pro-inflammatory cytokines

THP-1 cells were incubated for 72 h with 10^{-7} M VD3 to induce expression of the CD14 cell surface antigen (James S.Y., Williams M.A., Kelsey S.M., Newland A.C., and Colston K.W., 1997, *Biochem. Pharm.* **54**: p. 625-637). Triplicate samples of the differentiated or undifferentiated cells were challenged for 6 h with tenfold dilutions of LOS from the individual *Neisseria* strains or *Escherichia coli* endotoxin (strain 026:B6) (Sigma). A range of concentrations from 1 pg ml^{-1} to 100 ng ml^{-1} were examined in initial studies with LOS of the meningococcal immunotype strain L3. A concentration of 100 pg ml^{-1} was used to assess the neutralising effects of pooled human serum or immune mouse serum. To determine the concentration to be used in the neutralising assays, human serum or immune mouse serum was serially diluted and tested using WCE to assess binding of IgG to cells of NL1. A final dilution of 1 in 1,000 was used in the neutralization experiments (Braun J.M., Blackwell C.C., Poxton I.R., El Ahmer O., Gordon A.E., Madani O.M., Weir D.M., Giersen S., and Beuth J., 2002, *J.Infect.Dis.* **185**:p. 1431-1438).

Detection of cytokines

The ELISA to detect IL-6 and bioassay to detect TNFα reported previously were used in these studies (Braun J.M., Blackwell C.C., Poxton I.R., El Ahmer O., Gordon A.E., Madani O.M., Weir D.M., Giersen S., and Beuth J., 2002, *J.Infect.Dis.* **185**:p. 1431-1438).

Immune mouse sera

Strain NL1 was grown on HBA and killed by heating for 60 min at 100°C. The bacteria (10^9 in 100 µl) were injected in adjuvant free and pyrogen free saline (SIGMA) into the tail vene of three six week old male BALB/c mice on three consecutive days. This was followed by repeated intravenously inoculations with the same dose and batch of antigen at weeks 4, 8, 12 and 16. In week 20, LOS (100 µl, 100 ng ml^{-1}) obtained by hot phenol water extraction of NL strain was injected. Three days after the final injection, blood was collected

aseptically by cardiac puncture, allowed to clot, centrifuged at 500 x *g* for 15 min at 4°C. The supernatant was collected and diluted in pyrogen free saline (1 in 100). Complement was inactivated by heat treatment (56°C for 30 min) and the sera were stored in aliquots (1 ml) at -70°C. The production of antibodies was covered by an animal licence obtained from the British Home Office.

Antibodies to NL1 in samples taken from the mice before immunisation and at the end of the immunisation schedule were detected by WCE.

Statistical analysis

The mean, standard deviation (SD) and Student's t-test were calculated using Minitab for the Apple Mackintosh. To determine if the data were normally distributed, normal probability plots were used (Gardiner W.P., 1997, Statistics for the Biosciences. Prentice Hall Europe, Hertfordshire). Regression and analysis of variance showed that cytokine levels were normally distributed. Probability values were calculated with a confidence interval of 5% against the negative control treated with PBS only or the *E.coli* 026:B6 LPS. Two-sided analysis (5% confidence level) was carried out using a paired t-test for different endotoxin samples.

Results

In three independent experiments, the absorbed and unabsorbed pools were tested for bactericidal activity against 7 isolates of NL from the following countries: Scotland (2); Iceland (1); the Czech Republic (1); and Greece (3) (Table 4). The results obtained were consistent in each of the experiments. Eighteen meningococcal isolates, including the twelve immunotype reference strains, were also tested in the bactericidal assays (Table 5). The unabsorbed pool killed all the strains tested; the cfu of each strain was reduced by ≥ 80% that of their respective controls.

NL1 (Scotland)

Bactericidal activity against the following NL strains was absorbed by NL1: NL2 from Scotland; NL3 from Iceland; NL4 and NL5 from Greece; NL7 from the Czech Republic. One strain from Greece (NL6) was killed by the absorbed sera. Bactericidal activity against the following meningococcal strains was absorbed by *N. lactamica*1: immunotype reference strains C:NT:P1.2:**L1,8**, B:2a:P1.5,1.2:**L3**, C:11:P1.16:**L4**, B:4:P1.NT:**L5**, B:9:P1.1:**L7**, B:8,19:P1.7:**L8**, and A:21:P1.10:**L9**; B:15:P1.16 from England; B:15:P1.16 from Iceland; B:NT:NT, B:15:NT, from Scotland; B:2a:P1.2 from Greece.

NL7 (Czech Republic)

Bactericidal activity against the following NL strains was absorbed by NL7: NL1 from Scotland; NL3 from Iceland. Bactericidal activity against the following meningococcal strains was absorbed by NL7: immunotype reference strains C:NT:P1.2:**L1,8**, C:2c:P1.1:**L2**, B:5:1.7,1:**L6**, B:9:P1.1:**L7**, and B:8,19:P1.7:**L8**; B:15:NT and B:NT:NT from Scotland; and NG:4:NT from Greece. All other strains were killed by the absorbed serum pool.

NL3 (Iceland)

Bactericidal activity against the following NL strains was absorbed by NL3: NL1 and NL2 from Scotland; NL7 from the Czech Republic. Bactericidal activity against the following meningococcal strains was absorbed by NL3: immunotype reference strains C:2c:P1.1:**L2**, B:5:P1.7,1:**L6**, and B:9:P1.1:**L7**; B:15:NT and B:NT:NT from Scotland; NG:4:NT from Greece.

NL6 (Greece)

Bactericidal activity against NL4, one of the other Greek isolates, was absorbed by NL6. Bactericidal activity against the following meningococcal isolates was absorbed by NL6: immunotype reference strain B9:P1.1:**L7**; and

the Greek carrier isolate NG:4:NT. All other strains were killed by the unabsorbed and the absorbed serum pools.

Table 3: *N. meningitidis* immunotypes reference strains

Strain	Code	Major LOS	Minor LOS
C:NT:P1.2	126E	L1	*8*
C:2c:P1.1	35E	L2	*3,7,9*
B:2a:P1.5, 2	6275	L3	8
C:11:P1.16	89I	L4	
B:4:P1.NT	M981	L5	3,7,9
B:5:1.7, 1	M992	L6	
B:9:P1.7, 1	6155	L7	*3, 8*
B:8:P1.7, 1	M978	L8	*3, 4,7*
A:21:P1.10	120M	L9	*6, 8*

Table 4: Absorption of bactericidal antibodies of adult human pooled serum by *N. lactamica*

Phenotype	Origin		NL1	NL3	NL6	NL7
NG:NT:NT	Scotland	*N. lactamica* 1	+	+	-	+
NG:NT:NT	Scotland	*N. lactamica* 2	+	+	-	-
NG:NT:NT	Iceland	*N. lactamica* 3	+	+	-	+
NG:NT:NT	Greece	*N. lactamica* 4	+	-	+	-
NG:NT:NT	Greece	*N. lactamica* 5	+	-	-	-
NG:NT:NT	Greece	*N. lactamica* 6	-	-	+	-
NG:NT:NT	Czech Republic	*N. lactamica* 7	+	+	-	+

Table 5: Absorption of bactericidal activity against meningococcal strains by *N. lactamica* isolates from different parts of Europe

Phenotype	Origin	NL1	NL3	NL6	NL7
B:15:P1.7,16	England	+	-	-	-
C:2a:P1.2	Greece	+	-	-	-
NG:4:NT	Greece	-	+	+	+
B:15:P1.7,16	Iceland	+	-	-	-
B:15:NT	Scotland	+	+	-	+
B:NT:NT	Scotland	+	+	-	+
C:NT:P1.2: L1,8	USA	+	-	-	+
C:2c:P1.1: L2	USA	-	+	-	+
B:2a:P1.5,1.2:L3	USA	+	-	-	-
C:11:P1.16:L4	USA	+	-	-	-
B:4:P1.NT:L5	USA	+	-	-	-
B:5:1.7,1:L6	USA	-	+	-	+
B:9:P1.1:L7	USA	+	+	+	+
B:8,19:P1.7:L8	USA	+	-	-	+
A:21:P1.10:L9	USA	+	-	-	-

Inflammatory responses to LOS of *N. lactamica*

Because NL1 absorbed bactericidal activity against the broadest range of NL and meningococcal strains, it was used in the following experiments to assess induction of inflammatory responses and neutralising activities of immune serum raised against the strain.

Initial experiments found that maximum levels of TNF and IL-6 were induced at 6 hours following exposure of the THP-1 cells to 100 pg ml^{-1} of the LOS preparation from the meningococcal immunotype strain L3.

TNFα responses to LOS of different species in the presence and absence of VD3

In 6 independent experiments, incubation of undifferentiated THP-1 cells with LOS (100 pg ml^{-1}) from immunotypes L3, L6, NL1 or *E. coli* endotoxin resulted in detection of low levels (50-92 IU ml^{-1}) of TNFα compared with cells incubated with PBS (Figure 2a).

Compared with levels obtained with the undifferentiated THP-1 cells, there was a significant increase in TNFα activity ($p<0.01$) for each of the LOS preparations with the VD3-differentiated cells (Figure 2b). All LOS samples showed significantly higher TNFα activity compared with the *E.coli* endotoxin. With the VD3 differentiated cells, the highest TNFα levels were obtained with LOS from the L3 immunotype. TNFα levels for NL1 and *E.coli* were significantly lower than those elicited by LOS meningococcal immunotype strains L3 or L6.

IL-6 responses to LOS of different species in the presence and absence of VD3

A similar pattern was observed for induction of IL-6. Compared with cells incubated with PBS, incubation of undifferentiated THP-1 cells with LOS from the different strains resulted in low levels of IL-6 production (Figure 3a). Compared with IL-6 levels obtained with the undifferentiated THP-1 cells, there was a significant increase in IL-6 levels for each of the LOS preparations for the differentiated cells ($p<0.01$) (Figure 3b). All meningococcal LOS preparations induced significantly higher levels of IL-6 compared with the *E. coli* endotoxin (Figures 3b). NL1 LOS and *E. coli* LPS elicited IL-6 levels significantly lower than those obtained with LOS from the L3 ($P<0.01$).

Cytokine levels following treatment of LOS with pooled human serum

The pooled human serum from the bactericidal assays was incubated at a dilution of 1 in 1000 with LOS from the meningococcal immunotypes and LOS

from NL1. The serum pool significantly reduced TNF and IL-6 responses elicited by each of the LOS preparations tested. (Figures 4b and 5b)

Cytokine levels following treatment of LOS with immune mouse serum induced by the NL1 strain

In six experiments, TNFα and IL-6 levels for LOS from the following meningococcal immunotypes co-incubated with immune serum of mice vaccinated with strain NL1 were lower compared with cytokine levels obtained with LOS in the absence of serum: L2 ($P<0.01$); L3 ($P<0.01$); L7 ($P<0.01$); L8 ($P<0.01$); L9 ($P<0.01$); L11 ($P<0.01$). TNFα levels were lower for immunotypes L5 ($P<0.01$) and L10 ($P<0.01$), but IL-6 levels for these immunotypes were not significantly reduced ($P>0.13$). IL-6 levels were significantly lower for immunotype L6 ($P<0.01$), but TNFα levels were not significantly reduced ($P=0.34$). Cytokine levels for immunotype L4 were not significantly lower ($P=0.27$). Treatment of *E. coli* LPS with the immune mouse serum induced by NL1 reduced TNFα and IL-6 levels by not more than 15% (Figures 4c and 5c). The non-immune serum did not reduce cytokine levels in any of the endotoxin samples ($P=0.89$).

Detection of blood group or immunotype antigens on NL from different sources

The binding of blood group antigens and LOS immunotypes to NL isolates from the Czech Republic, from Russian immigrant children in Greece, from an Icelanding NL strain, and isolates from Scotland were assessed by WCE. The binding of blood group antibodies (Table 6) and meningococcal immunotype antibodies are summarised by country (Table 7) and by region of Greek NL isolates (Table 8).

Table 6: Binding of antibodies to human blood group antigens by *N. lactamica* isolates from different European countries

	Scotland	Iceland	Russian children	Czech Rep.

	n=12 No (%)	n=1 No (%)	n=27 No (%)	n=4 No (%)
P	1 (8.3)	1 (100)	10 (37)	1 (25)
P1	2 (16.7)	1 (100)	4 (14.8)	2 (50)
p^K	8 (66.7)	0 (0)	8 (29.6)	2 (50)
paragloboside	8 (66.6)	1 (100)	1 (3.7)	4 (100)
I	7 (58.3)	1 (100)	8 (29.6)	4 (100)

n.t., not tested)

Table 7: Binding of immunotyping antibodies by *N. lactamica* isolates from different European regions

	Scotland n=12 No (%)	Iceland n=1 No (%)	Russian children n=27 No (%)	Greek children n=73 No (%)	Czech Rep. n=4 No (%)
L1	1 (8.3)	0 (0)	4 (14.8)	24 (32.9)	1 (25)
L(3,7,9)	9 (75)	1 (100)	9 (33.3)	58 (79.5)	4 (100)
L8	2 (16.7)	0 (0)	4 (14.8)	7 (9.6)	2 (50)
L10	4 (33.3)	0 (0)	1 (3.7)	n.t.	1 (25)

n.t., not tested

Table 8: Binding of monoclonal antibodies to meningococcal immunotypes by *N. lactamica* strains isolated from different regions in Greece and isolates from Russian immigrant children

	FL	SR	SF	Greek children	Greece Russia
N	28	28	17	73	27
L1	7 (25)	10 (35.7)	7 (41.2)	24 (32.9)	4 (14.8)
L(3,7,9)	23 (82.1)	20 (71.4)	15 (88.2)	58 (79.5)	9 (33.3)
L8	3 (10.7)	2 (7.1)	2 (11.8)	7 (9.6)	4 (14.8)

Cz, Czech Republic; GRE Athens, Greece; ICE, Iceland; SCO, Scotland; SR, Serres; SF, Euros; SF, Florina; MC, *M. catarrhalis*; NL, *N. lactamica*

There was no significant difference in the number of isolates from the Czech Republic (n=4) and Scotland (n=12) expressing blood group antigens of the P- or Ii-system. Significantly fewer isolates from Russian immigrant children in Greece (n=27) expressed pK (P=0.039), paragloboside (P<0.001), and Ii (P=0.042) blood group antiges.

There was no significant differences in the distribution of LOS immunotype cross-reactivity between NL samples from the Czech Republic, native Greek children, and isolates from Scotland, except that significantly more isolates obtained from native Greek children bound antibodies to immunotype L1 compared to isolates obtained from other regions in Europe (Kruskal-Wallis analysis of variance by ranks). Meningococcal immunotypes L(3,7,9) (P<0.02) and L10 (P<0.01) was expressed by fewer NL isolates obtained from Russian immigrant children in Greece compared to isolates obtained from either Scotland, the Czech Republic, or from native Greek children. Meningococcal immunotypes L10 (P<0.01) was expressed by fewer NL isolates from Russian immigrant children in Greece compared to samples from either Scotland or the Czech Republic (Table 7). There were no significant differences in the immunotype phenotypes from NL isolates isolated from native Greek children in the regions of Serres, Euros, or Florina (Table 8).

Binding of cross-reactive antibodies obtained from BALB/c mice immunised with whole bacteria cells, LOS and/or OMV obtained from NL isolates

The development of cross-reactive antibodies via intravenous (i.v.) administration of whole cells (10^9bacteria), LOS (50 ng) or OMV (100 ng) obtained from commensal NL isolates were assessed in the murine BALB/c model. Immune mouse sera were assessed for cross-reactive anti-LOS antibodies by WCE against heat denaturated OMV obtained from meningococcal immunotype reference strains L1 (126E), L3 (6275), L7 (6155), L8 (M978), and L9 (120M) (Table 9). The induction of cross-reactive antibodies after i.v. administration correlated with the expression of immunotype phenotypes (Table 9) and was independent of the meningococcal

protein phenotypes. There was no significant differences in the induction of cross-reactive antibodies between whole bacteria, LOS or OMV obtained from NL vaccine isolates.

Route of administration:

Mucosal administration (oral, nasal, and/or through ear drops), intravenous and/or (sub)cutaneous application are effective ways to induce a lasting immunresponse. Vaccine candidates will be administered directly through solutions, sprays, and/or tablets, and/or through oral administration via vaccine candidates protected with a stomach resistant coating and/or capsule.

Table 9: Presence of cross-reactive serum antibodies after i.v. administration of whole bacteria, LOS or OMV in BALB/c mice

NL Strain	L1 126E	L3 6275	L7 6155	L8 M978	L9 120M
Immunotyp	L1,8	L(379),8	L(379),8	L(379),8,4	9,6,8
NL7; Cz4,G03	+	+	+	±	+
NL1; L01,G01	±	+	+	+	+
NL13; SR95	-	+	+	±	+
NL10; SR139	-	+	+	±	+
NL11; SR319	+	+	+	+	+
NL12; FL671	±	+	+	+	+
NL3; ICE,G02	-	+	+	±	+
GRE619	+	+	+	+	+
MC151	+	+	+	+	±
MC158	+	+	+	+	±
MC166	+	+	+	+	+
MC179	+	+	+	+	+
MC180	+	+	+	+	+

Data: +, presence of antibodies; -, absence of antibodies; ±, weak induction of functional antibodies compared to the homologous strain

Discussion of the experimental results

The absorption studies show that antigens found on commensal *Neisseriae* share antigens found on pathogenic meningococci that, surprisingly, are independent on the protein phenotype of commensal bacteria and meningococci. The unexpected variation of phenotypes found in *Neisseria lactamica* strains from different European regions provides evidence of a novel factor that absorbs bactericidal antibodies from normal human adult sera.

While endotoxin from *Neisseria meningitidis* is associated with severity and fatality of meningococcal disease, the biological inflammatory activity of endotoxin from commensal *Neisseria lactamica* strains sharing cross-reactive antigens with meningococci show that the commensal LOS molecules are far less toxic compared to meningococcal endotoxin associated with disease. The inflammatory assays showed further, that serum from mice immunised with the commensal *N. lactamica* strains induces functional, meningococcal cross reactive and endotoxin neutralising antibodies. These findings provided evidence, that *N. lactamica* LOS are an effective vaccine inducing protective, antimeningococcal endotoxin antibodies and that antibodies induced by meningococcal and/or commensal *N. lactamica* LOS are an effective treatment in meningococcal induced endotoxic shock.

The experiments measuring the binding of human blood group and meningococcal endotoxin antibodies provide evidence, that cross-reactive antigens were identified as glycoconjugates, namely oligosaccharide antigens found on some strains of *Neisseria lactamica*. Phenotyping of blood group like antigens and meningococcal immunotyping antibodies show further, that meningococcal and commensal *N. lactamica* endotoxin share structural homologous oligosaccharide and core antigens.

Assessment of the role of *M. catarrhalis* on the induction of natural immunity to meningococcal disease

Material and Methods were applied as described above for the commensal *Neisseriae* species.

Results

Bactericidal assays

The unabsorbed serum pool killed all strains tested (>80 % killing).

MC1: In three independent experiments, MC1 absorbed bactericidal activity against MC2 and MC3 but not the other two MC isolates tested. (Table 10). MC1 absorbed bactericidal activity against 13/30 (43%) meningococcal isolates tested: immunotype reference strains L1, L4, L5, and L9 (Table 11); B:15:P1.7,16 from England; B:15:P1.7,16 and C:4:P1.15 from Iceland; B:2a:P1.2, B:15:NT and B:NT:NT from Scotland; B:2a:1.2, B:NT:P1.9 and B:4:P1.15 from Greece (Table 12).

MC2: In three independent experiments, MC2 absorbed bactericidal activity against MC1, the Greek NL4 and NL8 from Scotland (Table 10). It absorbed bactericidal activity against 5/30 (17%) meningococcal strains tested: B:2a:P1.2, and B:NT:NT from Scotland; B:2a:P1.2, B:NT:P1.9 and B:4:P1.15 from Greece. All the immunotype reference strains were killed by the sera absorbed with MC2 (Tables 11 and 12).

Table 10: Absorption of bactericidal activity against MC and NL isolates by MC1 and MC2 (results of 3 independent experiments)

Code	Source	MC1	MC2
MC1	Scotland	+	+
MC2	Scotland	+	+
MC3	Scotland	+	-
MC4	Scotland	-	-
MC5	Scotland	-	-

+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool

- Reduction in bactericidal activity < 80% compared with the unabsorbed pool

Table 11: Absorption of bactericidal activity against meningococcal immunotype reference strains by MC1 or MC2 (results of 3 independent experiments)

Phenotype	LOS oligosaccharide α chain	MC1	MC2
C:NT:P1.2: **L1**	NeuNAcα (2→3) Galα (1→4) Galβ (1→4) Glcβ	+	-
C:2c:P1.1:**L2**	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
B:2a:P1.5,2: **L3**	NeuNAcα (2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
C:11:P1.16: **L4**	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
B:4:P1.NT: **L5**	(2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
B:5:P1.7,1: **L6**	NeuNAcα (2→3) GalNAcβ (1→3) Galα (1→4) Glcβ	-	-
B:9:P1.7,1: **L7**	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
B:8,19:P1.7, 1:**L8**	NeuNAcα (2→3) Galβ (1→4) Glcβ	-	-
A:21:P1.1.10 :**L9**	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	+	-
A:21:P1.10: **L10**	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	-
A:21:P1.10: **L11**	Galβ (1→4) Galβ (1→4) Glcβ	-	-
A:21:P1.NT: **L12**		-	-

+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool

- Reduction in bactericidal activity < 80% compared with the unabsorbed pool

Meningococcal immunotypes are highlighted in bold.

Table 12: Absorption of bactericidal activity against meningococcal isolates from different geographic regions by MC1 or MC2 (results of 3 independent experiments)

Phenotype	No.	Origin	MC1	MC2
B:15:P1.7,16	A11	England	+	-
B:NT:P1.9	1766	Greece	+	+
B:NT:P1.13	PE255	Greece	-	-
NG:NT:NT	ST776	Greece	-	-
NG:NT:NT	P481	Greece	-	-
B:2a:P1.2	TH39	Greece	-	-
B:2a:P1.2	TH44	Greece	+	+
B:4:P1.15	A43	Greece	+	+
C:2a:P1.2	A14	Greece	-	-
C:4:NT	A26	Greece	-	-
NG:4:NT	A48	Greece	-	-
B:15:P1.7,16	B14	Iceland	+	-
C:4:P1.15	Ice155	Iceland	+	-
B:15:NT	99-1787	Scotland	+	-
B:2a:P1.2,5	Sto B	Scotland	-	-
B:NT:NT	99/760	Scotland	+	+
C:2a:NT	A25	Scotland	-	-
C:2a:P1.2	StoC	Scotland	-	-
B:2a:P1.2	SNMP	Scotland	+	+

+ Reduction in bactericidal activity ≥80% compared with the unabsorbed pool

- Reduction in bactericidal activity < 80% compared with the unabsorbed pool

Assessment of binding of blood grouping and meningococcal immunotyping antibodies

Clinical isolates of MC (n=126) were from our culture collection. The binding of blood group antibodies against P, P1, p^K, paragloboside, I and meningococcal immunotype L(3,7,9) were measured by WCE. Because of the large number of strains to be tested and the limited amount of reagents, WCE was used and only the L(3,7,9) monoclonal tested as this epitope is most likely to be the one involved in induction of protective antibodies against disease causing strains.

MC1 bound antibodies to P, P1, p^K and I; MC2 bound only antibodies to p^K. None of the two strains bound antibodies to paragloboside or L(3,7,9).

Most clinical isolates of MC bound one or more antibody to the following antigens (Table 13): P (12.7%); P1 (23.8%); p^K (63.5%); paragloboside (17.5%); I (19.0%); and L(3,7,9) (30.2%).

Table 13: WCE for binding of antibodies to blood group antigens and L(3,7,9) by *M. catarrhalis* strains

Antigen	Positive MC strains n=126, No (%)
P	16 (12.7)
P1	30 (23.8)
p^K	80 (63.5)
Paragloboside	22 (17.5)
I	24 (19.0)
No binding of blood group antibodies tested	33 (26.2)
L(3,7,9)	38 (30.2)

Binding of antibodies to blood group antigens by *M. catarrhalis* isolates from Scotland

The binding of blood group antibodies against P, P1, pK, paragloboside (n=126) and meningococcal immunotype L(3,7,9) (n=187) to clinical isolates of *M. catarrhalis* from our bacteria collection obtained from adults (n=100) and children (n=26 for blood group antibodies, or n=87 for meningococcal immunotype antibodies). Binding of antibodies to pK did not differ significantly between isolates obtained from adults or children, while a greater number of isolates obtained from children bound other blood group antibodies tested. (Table 14).

Table 14: Binding of antibodies to blood group antigens antibodies by *M. catarrhalis* strains obtained from adults (n=100) and children (n=26)

Antigen	Strains isolated from adults (n=100)	Strains isolated from children (n=26)	Total strains (n=126)
P	15 (15)	1 (3.8)	16 (12.7)
P1	28 (28)	2 (7.7)	30 (23.8)
pK	61 (61)	19 (73.1)	80 (63.5)
Paragloboside	21 (21)	1 (3.8)	22 (17.5)
I	23 (23)	1 (3.8)	24 (19.0)
No binding of blood group antibodies	25 (25)	6 (23.1)	33 (26.2)

Data: positive cells (%age of positive cells)

Binding of antibodies to meningococcal immunotype antigens by *M. catarrhalis* isolates from Scotland

Significantly more strains isolated from children (n=87) bound antibodies to the meningococcal immunotype L(3,7,9) associated to disease to isolates obtained from adults (n=100). Most isolates obtained from children (74.77%) bound antibodies to meningococcal immunotype L1, and only few (5.7%) bound immunotype L8 antibodies (Table 15).

Growth of THP-1 cells

For the phagocytosis assays, the THP-1 (ECACC, Lot/CB 98/K/018 33629) suspension (50 ml) was washed twice at 300 x *g* in warm RPMI-1640 assay medium supplemented with 1 mM L-glutamine. The cells were resuspended to a final cell concentration of $1x10^6$ ml^{-1} in assay medium and incubated at 37°C for 30 min. The assay medium contained Dulbecco's phosphate buffered saline (pH 7.4) supplemented with 5×10^{-3} M glucose, 9×10^{-4} M $CaCl_2$, and 5×10^{-4} M $MgSO_4$.

THP-1 cells were assessed by flow cytometry for expression of the cell surface markers .

Preparation of propidium iodide (PI) labelled bacteria

Bacterial strains: Meningococci and commensal strains from our cell collection were used in these studies. All strains were grown for 18 hr on human blood agar (HBA). The colonies were suspended in 4 ml 2% (v/v) PBS buffered paraformaldehyde and stained with Vindelov's propidium iodide (2.4) for 15 min at RT. The cells were washed three times by centrifugation in PBS.

Enumeration of fluorescent bacteria: Prior to use, aliquots of PI-labelled bacteria were counted and the mean fluorescence intensity assessed by flow cytometry. To assess the number of bacteria in relation to a known number of fluorescence alignment beads, a method modified from Antal-Szalmas *et al.* [1997] and Lebaron *et al.* [1998] was used. To account for day to day variation, the mean fluorescence intensity (MnI) of the fluorescent beads was adjusted daily to a signal reading of 500. The fluorescence intensity of the PI-labelled bacteria was measured using the FL3 log channel and the percentage and MnI recorded. Aliquots of labelled bacteria (10^8 ml^{-1}) were stored in the dark at 4°C for up to one month. Prior to use in the phagocytosis assay, the appropriate volume was removed and warmed to 37°C.

Phagocytosis assay

Sera and antibodies: Unabsorbed and absorbed preparations of pooled human serum obtained from consenting adult volunteers were used in this part of the study.

Opsonising of bacteria: The bacteria (100 µl, 10^8 ml^{-1}) were opsonised with 5% (v/v) of the unabsorbed or absorbed serum for 30 min at 37°C and washed twice in sterile PBS. The PI-labelled bacteria were diluted in opsonising buffer (OB) containing PBS supplemented with $CaCl_2$ (0.13 g) and $MgSO_4$ (0.12 g) per litre PBS.

Phagocytosis: THP-1 cells (10^6 ml^{-1}) were pre-warmed (37°C) in assay medium for 30 min. Opsonised bacteria (100 µl) were added to duplicate samples of THP-1 cells (1 ml) and incubated at 37°C at 100 rpm for 15 min in an orbital incubator (Gallenkamp). Phagocytosis was terminated by adding 3 ml ice cold PBS supplemented with 0.02% EDTA. To assess the optimal

number of PI-labelled bacteria, several ratios of bacteria : cells were tested (1:1 to 200:1) and bacteria bound and ingested measured. To quench fluorescence of adherent bacteria on the surface of the THP-1 cells, the suspension was washed once by centrifugation at 300 x *g* in 4 ml of ice cold PBS supplemented with trypan blue (3 mg l^{-1}). The pellet was re-suspended for flow cytometric analysis in 1 ml ice cold 2% (w/v) PBS buffered paraformaldehyde. The samples were stored on ice and analysed by flow cytometry within 1 hour.

Flow cytometric analysis

THP-1 cell population: THP-1 cells were gated around the FS and SS channels. These gates were used to measure the red fluorescence (logFL3) of phagocytosed PI-labelled bacteria. The percentage and MnI of the positive cell populations showing phagocytosis were recorded. A combination of the two-percent and mean-intensity method to discriminate positive population in flow cytometry were used.

Assessment of phagocytosis: The percentage of positive cells in the population (%) was multiplied by the mean fluorescence intensity (MnI) of the positive cell population to provide the mean ingestion index (II). The II was used to compare phagocytosis in populations of cells. The results were compared to the numbers of bacteria per cell determined by confocal and fluorescence microscopy. The same batch of fluorescence alignment beads (ImmunoCheck, Coulter) was used to calibrate the logFL1, logFL2 and logFL3 fluorescence intensity to 500 to account for day to day variability.

Following fixation, the suspension of cells and bacteria (50 µl) was prepared for confocal and fluorescence microscopy. Statistical analysis A two-sided paired Mann-Whitney test (confidence interval, 95%) was used to assess the data.

The role of phagocytosis in meningococcal disease

Some sub-classes of IgG have opsonic functions facilitating phagocytosis and intracellular killing of bacteria by neutrophils (PMN), monocytes, and macrophages through the complement receptor C3 (CD11/18) or IgG affinity receptors (CD16, CD32, CD64). In the absence of antibodies, meningococci can bind to blood group antigens (Lewis^x, Lewis^a) found on monocytes. They are ingested but avoid the classical intracellular killing mechanism of lysozyme release and oxidative burst which leads to intracellular survival. Opsonin-independent intracellular uptake followed by the oxidative burst can involve the binding of *Neisseria* species to receptors for vitronectin and fibronectin on PMN (CD51, CD41 and CD66).

Invading meningococci shed outer membrane vesicles (blebs) containing some proteins and PMN are able to phagocytose and kill opsonised meningococci, but they are not able to detoxify endotoxin and release the debris (egestate) of the killed meningococci approximately two hours after phagocytosis (Figure 9). Antibodies to meningococcal LOS found in normal human serum can neutralise the inflammatory responses to LOS, but they might also be opsonising in nature. While IgG1 and IgM are associated with bactericidal action of human serum, the presence of anti-meningococcal LOS IgG antibodies allows LOS to be detoxified successfully by monocytes through the IgG high affinity FcγRI receptor (CD64) eliciting some release of pro-inflammatory cytokine. Antibody dependent phagocytosis and neutralisation without elicting inflammation is thought to be mediated through the two low affinity IgG receptors FcγRII (CD32) and the FcγRIII receptor (CD16) expressed on natural killer cells, monocytes (FcγRIIIa) and PMN (FcγRIIIb) [Bredius *et al.*, 1994a & b].

Results

Detection of cell surface antigens on phagocytic cells

THP-1 cells did not bind antibodies to CD3, CD4, CD8, CD11c, CD14, CD16 or CD25 antibodies. They bound antibodies to the following antigens: CD11b;

CD11/18; CD15; CD41; CD45; CD51; CD64; CD77; and H blood group antigen (Table 15).

Table 15 Expression of cell surface markers on immature THP-1 cells (mean of 6 experiments, ±SD)

	% positive population
Control	1.0
CD4	4.06 ±1.5
CD8	1.76 ±0.9
CD11/18	99.3 ±0.4
CD11b	45.3 ±2.9
CD11c	5.94 ±1.5
CD14	4.3 ±0.8
CD15	67.5 ±2.1
CD16	1.53 ±0.4
CD25	5.09 ±2.4
CD41	52.5 ±2.8
CD45	87.8 ±6.1
CD51	59.7 ±2.1
CD64	57.9 ±3.1
CD77	37.7 ±4.4
H	80.0 ±8.1

Effect of quenching

To discriminate between bound and ingested bacteria, THP-1 cells were assessed by flow cytometry and fluorescence microscopy in the presence and absence of the quenching agent trypan blue (Figure 10). All strains tested showed similar ingestion indices in the absence of trypan blue. Quenching of bound bacteria resulted in a significant reduction of the ingestion index of NL1 ($P<0.024$), L7 ($P<0.01$) and B:NT:NT ($P<0.01$), but not MC1 ($P=0.13$). Most of MC1 (87.0%) were ingested, with NL1 showing slightly lower uptake (74.6%). The meningococcal immunotype reference strain L7 (38.1%) and the

carrier strain B:NT:NT (34.6%) were phagocytosed in significantly lower number compared to both NL1 and MC1 ($P<0.01$). Quenching reduced the autofluorescence of THP-1 cells (control).

In six independent experiments, the unabsorbed serum pool opsonised all meningococcal and commensal strains tested at a ratio of 50 bacteria : THP-1 cell. Compared to the unquenched samples, the meningococcal immunotype reference strains were ingested by THP-1 cells as follows (percentage in the quenched sample compared to the unquenched control = 100% ± standard error): L3 (41.3% ±4.2); L5 (42.7% ±6.2); L6 (41.9 ±5.3); L7 (38.1% ±7.2); L8 (44.7 ±4.9). The values for the commensal strains were nearly twice that of the meningococcal strains: NL1 (73.6% ±5.6), NL3 (78.1 ±7.7); NL7 (77.5 ±3.5); MC1 (87.0 ±3.3); MC2 (79.3 ±9.1); and MC27 (76.1 ±4.7). Pre-treatment of the bacterial strains with the absorbed complement source resulted in low levels of ingestion of all strains tested, less than 25% compared with the unabsorbed pool.

Enumeration of ingested bacteria

In eleven independent experiments the mean intensity was used to calculated the mean number of ingested bacteria per THP-1 cell (± standard error) (Table 16) using the regression equation described above .

Table 16 Mean number of (a) commensals or (b) meningococci ingested per THP-1 cell after 15 min incubation (mean of eleven independent experiments ± standard deviation)

(a)

NL1	NL3	NL7	MC1	MC2	MC27
25.1 ±4.2	25.5 ±2.9	19.6 ±2.2	29.8 ±4.5	33.3 ±2.0	20.2 ±2.0

(b)

L3	L5	L6	L7	L8	B:NT:NT
10.8	12.9	16.4	12.7	13.6	13.7

±3.9	±3.5	±1.2	±5.9	±1.8	±6.6

Analysis of differences in ingestion of individual strains by species

Mean number of ingested bacteria per THP-1 cell were grouped by species and analysed using two-sided non-parametric Mann-Whitney test (confidence at 95%) to compare differences between the mean number of opsonised meningococci and commensals ingested: NL mean=23.4, median=22.0, SD=10.66; MC mean=27.78, median=26.4, SD=11.38; NM mean=13.34, median=12.95, SD=13.75.

There were no significant differences between the number of ingested *N. lactamica* and *M. catarrhalis* (P=0.1335). Significantly greater number of NL (P<0.001) or MC (P<0.001) were ingested per THP-1 cell compared to group B meningococci.

The effect of antibody and complement on ingestion of commensal species and meningococci by THP-1 cells

N. lactamica

Non-opsonised NL1 and NL1 opsonised with the complement source were ingested at a constant rate reaching a maximum ingestion index of approximately 90 after 45 min. NL1 opsonised with the serum pool showed a linear increase in the ingestion index after 5 min reaching a maximum after 25 min (II=200). NL1 opsonised with the serum pool and the complement source showed a sigmoid relationship after 5 min reaching a plateau after 15 min.

N. meningitidis L7

Non-opsonised L7 and L7 opsonised with the complement source were ingested reaching a maximum ingestion index of approximately 30 after 45 min (Figure 11). L7 opsonised with the serum pool showed an increase in the ingestion index after 5 min reaching a maximum of 90 after 20 min. L7 opsonised with the serum pool and the complement source reached a maximum ingestion index of 90 after 15 min. Time curves for other

commensals and meningococci yielded similar results with NL1 representing commensal NL and MC strains, and L7 representing meningococci.

Absorption experiments

Three independent experiments with unabsorbed and absorbed sera are summarised in Table 17. The ingestion index of the unabsorbed pool co-incubated with the complement source was given a value of 100 and the ingestion index obtained with the absorbed serum is presented as a percentage of the control. A reduction in ingestion index of more than 50% compared with the unabsorbed serum pool was scored as negative (-) reflecting significant reduction in opsonising activity. A reduction of 25 – 50 % was scored as partly absorbed (↓). A reduction of less than 25 % was considered to be positive (+) for opsonic activity. None of the absorbed samples eliminated phagocytic activity completely which indicates that bacteria and/or cell receptors other than immunoglobulin receptors were involved in binding and ingestion of the bacteria.

Table 17: Ingestion indices for phagocytosis of (a) meningococcal immunotype strains and (b) *N. lactamica*, *M. catarrhalis* with the unabsorbed pool and samples of the pool absorbed with meningococcal immunotypes or commensal species (mean of three independent experiments)

(a)

Test strain	L3		L5		L6		L7		L8	
	%	S	%	S	%	S	%	S	%	S
Unabsorbed pool	100	+	100	+	100	+	100	+	100	+
Absorbed with NL1	61	↓	12	-	96	+	69	↓	7	-
Absorbed with NL3	89	+	91	+	71	↓	57	↓	92	+
Absorbed with NL7	94	+	93	+	55	↓	93	+	99	+
Absorbed with	88	+	92	+	97	+	95	+	96	+

MC1										
Absorbed with MC2	96	+	87	+	98	+	95	+	95	+
Absorbed with MC27	71	↓	94	+	95	+	96	+	92	+
Absorbed with L3	8	-	12	-	53	↓	8	-	73	↓
Absorbed with L7	9	-	13	-	92	+	8	-	69	↓
Absorbed with L8	67	↓	15	-	91	+	57	↓	9	-

(b)

Test strain	NL1		NL3		NL7		MC1		MC2		MC27	
	%	S	%	S	%	S	%	S	%	S	%	S
Unabsorbed pool	100	+	100	+	100	+	100	+	100	+	100	+
Absorbed with NL1	3	-	5	-	65	↓	95	+	99	+	93	+
Absorbed with NL3	11	-	7	-	4	-	72	↓	97	+	98	+
Absorbed with NL7	6	-	4	-	3	-	94	+	93	+	95	+
Absorbed with MC1	96	+	18	-	94	+	8	-	11	-	91	+
Absorbed with MC2	92	+	92	+	97	+	13	-	9	-	95	+
Absorbed with MC27	93	+	95	+	94	+	91	+	91	+	4	-
Absorbed with L3	55	↓	68	↓	57	↓	87	+	98	+	72	↓
Absorbed with L7	61	↓	52	↓	70	↓	91	+	94	+	98	+
Absorbed with L8	7	-	18	-	63	↓	89	+	93	+	91	+

+ opsonic activity, - no activity ≤25% of unabsorbed pool, ↓ reduction in opsonic activity by 25%>75% of unabsorbed pool

Absorption with *N. lactamica* strains

Absorption with the Scottish strain **NL1** reduced opsonic activity against the following strains: L3 (61%); L5 (12%); L7 (69%); L8 (7%); NL1 (3%); NL3 (5%); NL7 (65%). Absorption with the Icelandic strain **NL3** reduced opsonic activity against the following strains: L6 (71%); L7 (57%); MC1 (72%); NL1 (11%); and NL3 (8%). Absorption with the Czech strain **NL7** reduced opsonic activity against the following strains: L6 (55%); NL1 (6%); and NL3 (4%); NL7 (3%).

Absorption with *M. catarrhalis* strains

MC1 reduced opsonic activity against NL3 (18%), MC2 (11%) and the homologous strain MC1 (4%). **MC2** reduced the activity against MC1 (13%) and the homologous strain MC2 (9%) only. **MC27** reduced opsonic activity against the meningococcal immunotype L3 (71%) and the homologous strain MC27 (4%).

Absorption with *N. meningitidis* strains

Absorption of the pool with immunotype **L3** reduced opsonic activity against the following strains: L3 (8%); L5 (12%); L6 (53%); L7 (8%); L8 (73%); NL1 (55%); NL3 (68%); NL7 (57%); and MC27 (73%). Absorption with immunotype **L7** reduced opsonic activity against the following strains: L3 (9%); L5 (13%); L7 (8%); L8 (69%); NL1 (61%); NL3 (52%); and NL7 (70%). Absorption with immunotype **L8** reduced opsonic activity against the following strains: immunotypes L3 (67%); L5 (15%); L7 (57%); L8 (9%); the commensal strains NL1 (7%); NL3 (18%); and NL7 (63%).

Discussion

The method used was developed to attempt to reduce some of the problems assosiated with the measurement of phagocytosis: donor variability, different stages of monocyte activation or maturation, differences in the phagocytic response within an PBMC population, the presence of abnormal non-specific functions of PBMC, or contamination with leukocytes or lymphocytes. Flow

cytometry provided an objective, rapid method providing semiquantitiative estimates of the numbers of bacteria per phagocyte. Quenching with trypan blue allowed discrimination between surface bound and ingested bacteria.

The high affinity IgG receptor (FcγRI) (CD64) associated with immune phagocytosis is constitutivly expressed on THP-1 cells, as is the complement receptor (C3bR) (CD11/18). The bacteria count using the flow cytometric method was in general agreement with the enumeration using the Thoma counting method. A linear relationship (r^2=96.6%) was observed between the number of bacteria phagocytosed and the MnI at ratios between 4:1 and 75:1 bacteria per THP-1. Quenching of bound but not ingested bacteria was an effective way to assess intracellular uptake of PI-labelled bacteria to THP-1 cells. Differences in the binding and ingestion were observed between commensals and group B meningococci. THP-1 cells bound a similar number of all strains tested (approximately 30 – 40 bacteria per cell). THP-1 cells ingested greater numbers of commensal strains after 15 min (20.2 – 33.3) compared to group B meningococci (10.8 – 16.4) (P<0.01).

All commensal and meningococcal strains showed similar time curves for ingestion of opsonised bacteria. Maximal ingestion of opsonised bacteria with pooled human serum and complement was observed after 15 – 20 min; 75% of bacteria were ingested after 7.5 min. Differences in the number of bacteria ingested depended on the availability of human serum. Low levels of phagocytosis occurred when commensals or meningococci were incubated in the absence of serum or in the presence of the complement source. The kinetics of phagocytosis of serum opsonised bacteria in the presence or absence of complement suggest that both IgG and complement receptors might be involved in opsonophagocytosis of meningococci and commensal species.

These findings are suggest that successful phagocytosis of meningococci depend on the presence of human subclasses of IgG and/or complement.

Effect on phagocytosis of absorption of pooled human serum by commensals and meningococci

Opsonophagocytic activity of homologous strains was absorbed by all strains tested.

N. lactamica: All NL strains tested reduced opsonophagocytic activity of the pooled serum against the NL strains tested. Phagocytosis of MC1 was reduced with serum absorbed with NL3. Activity against meningococcal imunotypes L3, L5 and L8 was reduced by absorption with NL1. Phagocytosis of L6 was reduced with the serum absorbed with NL3 or NL7 and phagocytosis of L7 was reduced with serum absorbed with NL1 or NL3. Opsonophagocytic activity against other strains was not affected by absorption with NL strains.

M. catarrhalis: Although absorption with MC1 removed bactericidal activity against a number of meningococcal strains, the absorbed sera still contained opsonising anctivity. None of the MC isolates absorbed opsonins for the NL strains tested. MC1 absorbed opsonins against MC2 and MC2 absorbed opsonins against MC1. Absorption of the serum with MC27 reduced phagocytosis of meningococcal immunotype L3. All other strains were not affected by absorption with MC.

N. meningitidis: Opsonophagocytic activity for immunotypes L3, L5 and L7 was absorbed by Immunotypes L3, L7, and L8. Opsonins for L3 and L8 were absorbed by immunotype L3. Activity against NL1 and NL7 was reduced by absorption with L3, L7 or L8. Absorption of serum with L7 or L8 reduced phagocytosis of NL3. Absorption with immunotype L3 reduced opsonising activity against MC27. All other strains were not affected by absorption with meningococci.

Complement-dependent bactericidal activity of pooled human serum correlated with opsonophagocytic activity in most cases (Table 18). These findings suggest that antibodies found in normal human serum can be both bactericidal and/or opsonic in nature.

Table 18 Comparison of bactericidal (B) and opsonic (O) activities against (a) meningococcal immunotype reference strains, and (b) commensal species for human pooed serum absorbed by commensal species

(a)

Test strain	L3		L5		L6		L7		L8	
Serum absorbed with	B	O	B	O	B	O	B	O	B	O
Unabsorbed serum	+	+	+	+	+	+	+	+	+	+
NL1	-	↓	-	-	+	+	-	↓	-	-
NL3	+	+	+	+	-	↓	-	↓	+	+
NL7	+	+	+	+	-	↓	-	+	-	+
MC1	+	+	-	+	+	+	+	+	+	+
MC2	+	+	+	+	+	+	+	+	+	+
MC27	+	↓	+	+	+	+	+	+	+	+
Correlation coefficient	0.645		0.645		1		0.73		0.645	

(b)

Test strain	NL1		NL3		NL7		MC1		MC2		MC27	
Serum absorbed with	B	O	B	O	B	O	B	O	B	O	B	O
Unabsorbed serum	+	+	+	+	+	+	+	+	+	+	-	+
NL1	-	-	-	-	+	↓	+	+	+	+	-	+
NL3	-	-	-	-	-	-	+	↓	+	+	-	+
NL7	-	-	-	-	-	-	+	+	+	+	-	+
MC1	+	+	-	-	+	+	-	-	-	-	-	+
MC2	+	+	+	+	+	+	-	-	-	-	-	+
MC27	+	+	-	+	-	+	+	+	+	+	-	-
Correlation coefficient	1		0.73		0.417		0.730		1		na	

+, bactericidal or opsonising activity

-, absence of bactericidal or opsonising activity

Avoidance of phagocytosis by NM as a virulence factor

Meningococci possess virulence factors associated with avoidance of opsonisation, phagocytosis or intracellular killing. Over-expression of capsular polysaccharide shields epitopes on the bacterial cell surface from antibodies and complement and is associated with increased bacterial survival *in vivo*

Sialyl-LOS phenotypes are important not only in evading the complement cascade but also in resisting complement and anti-LOS antibody mediated phagocytosis. The presence of the sialylated LOS phenotypes found on invasive meningococci is linked to the ability of these strains to evade complement mediated killing by masking the immunoactive terminal galactose residue of some immunotypes .

Passive Immunisation

Acute meningococcal disease is characterized by the release of inflammatory mediators and cytokines. Patients with severe meningococcal meningitis and septicaemia lack sufficiant levels of bactericidal, opsono-phagocytic and anti-inflammatory cytokines directed against meningococcal glycoconjugates and lipo-oligosaccharides (LOS). In the absence of these antibodies meningococci, meningococcal cell fragments and membrane vesicles and/or blebs are ingested by monocytes, macrophages and/or granulocytes via non-specific scavenger receptors, and/or lectins, and/or LOS binding receptors (i.e. CD14), and/or other phagocytose and/or endocytosis associated receptors (i.e. CD51, CD61, CD66 receptor families), followed by intracellular degradation, and release of pro-inflammatory mediators that can be detected in large amounts in the serum and/or neural fluids (Figure 9).

In the presence of anti-endotoxin antibodies and/or antibodies directed against other cell surface and/or capsular antigens the inflammatory response, measured by the levels of released cytokines (Figures 2-7), is significantly reduced, while phagocytic activity is higher in the presence of opsono-

phagocytic antibodies with variations in phagocytic aktivity and serum IgG concentration between individual donors.

The effect of functional antibodies effective against meningococcal LOS is summarized in Table 19. Monoclonal antibodies produced by immunisation of BALB/c or CD-1 mice with meningococcal endotoxin, or LOS obtained from commensal species (Table 19.a) show bactericidal, opsono-phagocytic and anti-inflammatory properties. Antibodies directed against human blood group antigens showing homology with meningococcal immunotypes and commensal endotoxins (Table 20) were effective anti-inflammatory and opsono-phagocytic agents, but partially lacked bactericidal activity (Table 19.b). These antibodies apperar to be effective agents to reduce the severety of meningococci, meningococcial endotoxin, membrane blebs and vesicles or cell debrie observed during acute meningococcal disease (Figure 9).

In addition, the anti-inflammatory function of all antibodies tested (Figure 12) was significantly reduced in the present of sodium-selenite, in monocytes and granulocytes incubated with sodium selenite prior to or after LOS challenge, or in the absence of antibodies but co-incubation with sodium selenite. These findings provide evidence that a passive immunisation shedule using anti-meningococcal-LOS or anti-blood group antibodies in the presence or absence of sodium selenite is a potential treatment during meningococcal disease, and a potential preventive measure for close contacts and/or susceptible individuals.

Table 15: Binding of antibodies to blood group antigens and meningococcal immunotype antibodies by *M. catarrhalis* strains obtained from adults (n=100) and children (n=26)

Antigen	Strains isolated from adults (n=100)	Strains isolated from children (n=87)
L1	n.t.	65 (74.7)
L(3,7,9)	23 (23)	32 (36.8)
L8	n.t.	5 (5.7)

Data: positive cells (%age of positive cells); n.t.: not tested

Inflammatory response of LOS from meningococci and MC isolates

In six independent experiments in which each control and test condition was carried out in triplicate, VD3 differentiated THP-1 cells were challenged with LOS (100 pg ml^{-1}) from meningococcal immunotypes L3, L6, from MC1, MC2 and *E. coli* endotoxin. LOS from the L3 immunotype induced significantly higher TNFα levels compared to TNFα levels obtained with LOS from MC1, MC2 and *E.coli* (**P<0.01**) (Figure 6). MC2 induced significantly lower levels of TNFα compared to immunotype L3, L6 and MC1 but not *E. coli* LPS (**P<0.05**).

In six independent experiments, a similar pattern was observed for induction of IL-6. All except LOS from MC2 induced significantly higher levels of IL-6 compared with the *E. coli* LPS (Figures 7). MC2 LOS preparations and *E. coli* LPS elicited IL-6 levels significantly lower than those obtained with LOS from meningococcal immunotype strains L3, L6, and MC1 (**P<0.01**).

Discussion

The absorption studies show that antigens found on *M. catarrhalis* strains share, and induce functional and bactericidal antibodies against antigens found on pathogenic meningococci. Similar to commensal *Neisseriae* strains, endotoxin obtained from some *M. catarrhalis* strains induces significantly lower cytokine levels compared to meningococcal endotoxin immunotype L(3,7,9). The endotoxin moieties were identified to share structural and antigenetic homology with human blood group and meningococcal endotoxin antigens, and are, similar to commensal *Neisseriae*, the main molecule with cross-reactive antigenicity to meningococcal endotoxins.

M. catarrhalis isolates are commonly found as commensal strains in the nasopharynx of young children, as well are a common infectious agent for childhood otitis media. The experiments presented provide evidence that the carriage of, or infection with *M. catarrhalis* induces functional antibodies directed against meningococcal endotoxin, and that endotoxin obtained from *M. catarrhalis* is a vaccine for the protection against meningococcal disease.

Table 19: Assessment of functional monoclonal and polyclonal antibodies obtained from a.) BALB/c mice immunized with LOS based vaccies, b.) BALB/c mice immunized with human blood group antigens, and c.) human serum absorbed with N. lactamica immunotype L(3,7,9), N. lactamica or M. catarrhalis

a.

	anti-L(3,7,9) mAb (IgG, IgM, IgA)	anti-LOS Ab's from *N. lactamica* (IgG, IgM, IgA)	anti-LOS Ab's from *M. catarrhalis* (IgG, IgM, IgA)
bactericidal	yes	yes	yes
opsono-phagocytic	yes	yes	yes
anti-inflammatory	yes	yes	yes
cold-agglutination	no	no	no

b.

	anti-paragloboside pAb and mAb [1)] (IgG, IgM, IgA)	anti-Ii pAb and mAb [1)] (IgG, IgM, IgA)	anti-pK pAb and mAb [1)] (IgG, IgM, IgA)
bactericidal	yes	no	no
opsono-phagocytic	yes	yes	yes
anti-inflammatory	yes	yes	yes

cold-agglutination	no	yes	no

c.

	human serum pAb (IgG, IgM, IgA)	human serum absorbed with L(3,7,9) LOS (IgG, IgM, IgA)	human serum absorbed with *N. lactamica* (IgG, IgM, IgA)	human serum absorbed with *M. catarrhalis* (IgG, IgM, IgA)
bactericidal	yes	no	no	no
opsono-phagocytic	yes	no	no	no
anti-inflammatory	yes	no	no	no

[1)] sialylated and non-sialylated forms

mAb = monoclonal antibodies obtained from fusion of spleen lymphocytes from BALB/c mice immunised with the antigens with a human and/or animal hybridoma cell line

pAb = polyclonal antibodies obtained from serum of BALB/c mice immunised with the antigens

IgA = immunglobulin A

IgG = immunglobulin G_{1-4}

IgM = immunglobulin M

Table 20: Oligosaccharide structures common to human blood group antigens and oligosaccharide chains of LOS of *N. meningitidis*, *M. catarrhalis* and commensal species

LOS	Terminal oligosaccharide α chain oligomer of the G1 region
L1 L11 p^k, CD77 MC IV P1 blood group MC VII	Galα (1→4) Galβ (1→4) Glcβ Galα (1→4) Galβ (1→4) Glcβ Galα (1→4) Galβ (1→4) Glcβ Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galα (1→4) Galβ (1→4) GlcNAcα (1→2)Glcβ
L8 Cer- dihexocide MC III	Galβ (1→4) Glcβ Galβ (1→4) Glcβ Galβ (1→4) Glcα (1→2) Glcβ
P globoside L6	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ GalNAcβ (1→3) Galα (1→4) Glcβ
I c β adult blood group I d αβ adult blood group MC VI	Galβ (1→4) GlcNAcβ (1→6) GalNAcβ Galβ (1→4) GlcNAcβ (1→6) GalNAcβ Galβ (1→4) GlcNAcα (1→2) Glcβ
L2 L3 i b foetal blood group I b α adult blood group L4 L5 L7 L9 L10 Paragloboside i a foetal blood group I a αβ, I b β adult blood group I c α adult blood group	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Sialyl→ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Sialyl→ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Sialyl→ Galβ (1→4) GlcNAcβ (1→3; 1→6) Galβ (1→4) GlcNAcβ (1→4) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3; 1→6) Galβ (1→4) GlcNAcβ (1→4) Galβ (1→4) Glcβ Galβ (1→4) GlcNAcβ (1→3) Galβ (1→6) GalNAcβ
MC V	GlcNAcα (1→2) Glcβ
MC I	Glcβ
MC II	Glcα (1→2) Glcβ

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine ; Glc, glucose; NeuNAc (Sialyl), sialyc acid

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